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# Dosage-dependent switch from G protein-coupled to G protein-independent signaling by a GPCR

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**G-protein-coupled receptors (GPCRs) mostly signal through heterotrimeric G proteins. Increasing evidence suggests that GPCRs could function in a G-protein-independent manner. Here, we show that at low concentrations of an agonist, $\beta_2$-adrenergic receptors ($\beta_2$-ARs) signal through $G\alpha_s$ to activate the mitogen-activated protein kinase pathway in mouse embryonic fibroblast cells. At high agonist concentrations, signals are also transduced through $\beta_2$-ARs via an additional pathway that is G-protein-independent but tyrosine kinase Src-dependent. This new dosage-dependent switch of signaling modes of GPCRs has significant implications for GPCR intrinsic properties and desensitization.**

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## Introduction

In a classical G-protein-coupled receptor (GPCR) signaling pathway, GPCRs directly relay the signals by activating heterotrimeric guanine nucleotide binding regulatory proteins (G proteins) (Gilman, 1987). Based on sequence homologies and functional similarities of their $\alpha$ subunits, these G proteins are grouped into four families: $G_s$, $G_i$, $G_q$, and $G_{12}$ (Simon *et al*, 1991). Src-family tyrosine kinases are another major group of cellular signal transducers and have been demonstrated to directly relay signals from membrane receptors (Thomas and Brugge, 1997). Many GPCRs are able to increase the activity of Src-family tyrosine kinases (Chen *et al*, 1994; Ishida *et al*, 1995; Ptasznik *et al*, 1995; Luttrell *et al*, 1996; Rodriguez-Fernandez and Rozengurt, 1996; Schieffer *et al*, 1996; Wan *et al*, 1996, 1997; Ma *et al*, 2000; Luttrell and Luttrell, 2004). Some of the documented GPCR-induced events that involve tyrosine kinases include the activation of mitogen-activated protein kinase (MAPK) cascades (Chen *et al*, 1994; Luttrell *et al*, 1996; Simonson *et al*, 1996; Wan *et al*, 1996, 1997; Schieffer *et al*, 1997).

For the Gs-coupled $\beta_2$-adrenergic receptors ($\beta_2$-ARs), c-Src has been shown to act downstream of Gs to mediate $\beta_2$-AR activation of ERK MAPK (the extracellular signal-regulated kinase subfamily of MAPKs) in some cell types. The molecular mechanisms by which c-Src participates in these pathways seem to differ, depending on cell types and even clonal variants of the same cell type (Lefkowitz *et al*, 2002). For example, in HEK-293 cells, it was reported that, after ligand stimulation of transfected $\beta_2$-AR, β-arrestin formed a complex with Src and brought Src to the $\beta_2$-AR, leading to receptor desensitization/internalization, which initiates a second wave of signaling including the ERK MAPK pathway (Luttrell *et al*, 1999). It was also suggested that, after $\beta_2$-AR activation of Gs and adenylyl cyclase (AC), PKA phosphorylated $\beta_2$-AR and enhanced $\beta_2$-AR's coupling to Gi protein. The $G\beta\gamma$ subunits released from Gi activated Src leading to Ras/c-Raf1/MEK/ERK activation (Daaka *et al*, 1997). On the other hand, other groups have reported that endogenous or transfected $\beta_2$-ARs, via $G\alpha_s$ activation of PKA, Rap1, and B-Raf, stimulated ERK in an Src-dependent and PTX (pertussis toxin)-insensitive manner (Schmitt and Stork, 2000, 2002a; Friedman *et al*, 2002). This discrepancy of results from HEK-293 cells was later attributed to the possible uses of different variants of HEK-293 cell lines (Lefkowitz *et al*, 2002). In other cell types such as CHO cells, PC12 cells, and NIH3T3 cells, the activation of ERK by Gs-coupled receptors seemed also to involve Gs, PKA, and Src proteins (Klinger *et al*, 2002). Furthermore, for the Gs-coupled $\beta_3$-AR, it was reported that c-Src and $\beta_3$-AR were co-immunoprecipitated in an agonist-dependent and PTX-sensitive manner (Cao *et al*, 2000). It was suggested that $\beta_3$-AR activated Gi, which activated c-Src. Activated c-Src was then recruited to $\beta_3$-AR by binding to $\beta_3$-AR (Cao *et al*, 2000).

In the above studies, the activation of c-Src by $\beta_2$-AR is G-protein-dependent. During our study of ERK activation by $\beta_2$-ARs in $G\alpha_s^{-/-}$ mouse embryonic fibroblast (MEF) cells, we noticed that there is a G-protein-independent pathway. We have pursued this G-protein-independent signaling in this current study. Although GPCRs are known to transduce signals through G proteins, there are indications that these receptors are also able to signal in a G-protein-independent manner (Milne *et al*, 1995; Ali *et al*, 1997; Brakeman *et al*, 1997; Sexl *et al*, 1997; Araki *et al*, 1998; Hall *et al*, 1998; Jin *et al*, 1998; Cao *et al*, 2000; Miller and Lefkowitz, 2001; Seta *et al*, 2002; Whistler *et al*, 2002; Bockaert *et al*, 2004; Shenoy *et al*, 2006; Wang *et al*, 2006). However, the mechanistic relationship between the G-protein-dependent and the G-protein-independent signaling by GPCRs, and the biochemical mechanism by which GPCRs initiate G-protein-independent signaling have not been elucidated. Here, we use the

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activation of the ERK MAPK pathway in MEF cells as a model system to investigate G-protein-dependent and -independent signaling by GPCRs. The reason for using MEFs was to take advantage of the availability of specific gene-knockout MEF cells. We found that the response of MAPK to stimulation by $\beta_2$-AR is biphasic. While the first phase of this response is abolished in $G\alpha_s$-deficient cells, deletion of Src-family tyrosine kinases eliminates the second phase. Furthermore, $\beta_2$-AR can directly activate Src, independent of $G\alpha_s$ and β-arrestins. Thus, the receptor signals are transduced through two mechanisms with agonist dosage acting as the switch.

## Results

### *Biphasic dose–response of $\beta_2$-AR activation of ERK MAPK*

In MEF cells, stimulation with isoproterenol, an agonist for $\beta_2$-AR, increased the kinase activity of ERK MAPK (Figure 1A). The dose–response curve of this stimulation was better fitted with a two-site competition equation than with a one-site competition equation, indicating the existence of two distinct phases of $\beta_2$-AR signaling (Figure 1B). The $EC_{50}$ of isoproterenol for the first phase was ~1 nM and the $EC_{50}$ for the second phase was ~1 μM. Pre-treatment of MEF

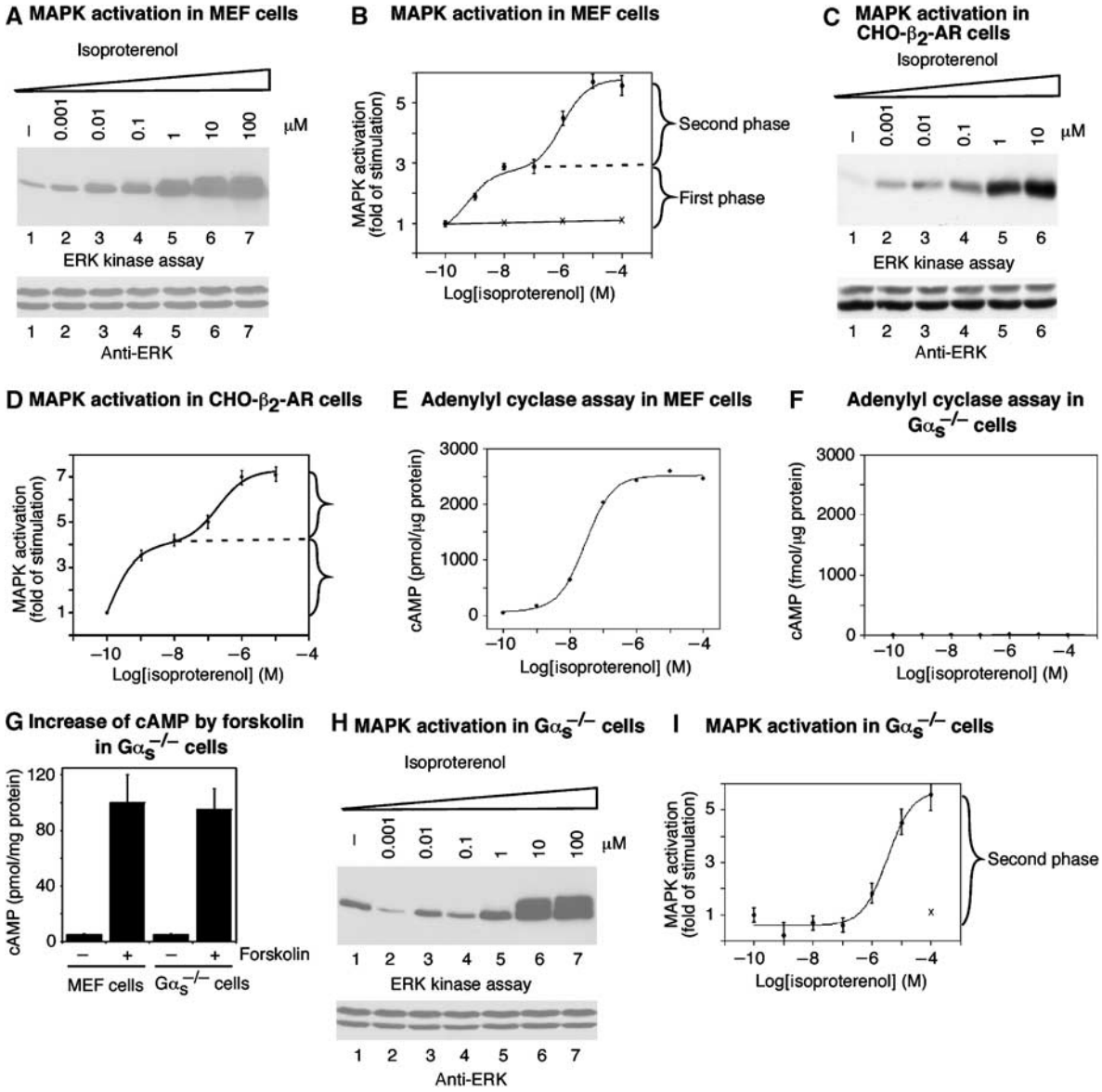


**Figure 1** $G\alpha_s$ mediates the first phase of the stimulation of ERK MAPK by $\beta_2$-AR. (**A**) Top: different concentrations of isoproterenol increased the kinase activity of ERK MAPK in MEF cells. Whole-cell lysates were prepared from MEF cells. Activated ERK MAPK proteins were immunoprecipitated from cell lysates by a monoclonal antibody against phospho-p44/42 ERK MAPK (crosslinked to agarose beads). The ERK MAPK activity was measured by the phosphorylation of substrate GST-Elk-1, which was detected by Western blotting with an anti-phospho-Elk-1 antibody. Bottom: Western blot with anti-ERK MAPK antibody showing that similar amounts of cell lysates were used in each lane. (**B**) The data in (A) were quantified and the stimulation of MAPK was shown in comparison to the basal (without isoproterenol treatment). The data in the presence of ICI-118551 are marked with x. Data represent mean ± s.d. of three experiments. (**C, D**) Dose–response of ERK activation by isoproterenol in CHO cells stably expressing human $\beta_2$-AR. (**E**) Dose–response of cAMP production by isoproterenol stimulation in MEF cells. (**F**) No stimulation of ACs by isoproterenol in $G\alpha_s^{-/-}$ cells. (**G**) Increase of cAMP by forskolin in $G\alpha_s^{-/-}$ cells. (**H, I**) Different concentrations of isoproterenol increased the kinase activity of ERK MAPK in $G\alpha_s^{-/-}$ cells. The data point in the presence of 10 μM PP2 is marked with x. Data represent mean ± s.d. of three experiments.

 

cells with selective $\beta_2$-AR antagonist ICI-118551 (1 μM) blocked the stimulation of ERK MAPK (both phases) by isoproterenol (Figure 1B). This biphasic dose–response of isoproterenol activation of ERK MAPK had been observed before in COS-7 cells, although it was not discussed and explored further (Crespo *et al*, 1995). Furthermore, we established a CHO cell line stably expressing $\beta_2$-AR. In these cells, the dose–response curve of $\beta_2$-AR activation of ERK was also biphasic (Figure 1C and D). Although there might be other explanations for this biphasic dose–response curve of $\beta_2$-AR activation of ERK MAPK, the simplest explanation is that $\beta_2$-AR signals through two different signaling pathways: at low concentrations ($< \sim 100$ nM) of isoproterenol, $\beta_2$-AR signals through one pathway; at high concentrations ($> \sim 100$ nM), $\beta_2$-AR triggers an additional pathway.

### *$G\alpha_s$ is responsible for the first phase of the response*

As $G\alpha_s$ is known to mediate $\beta_2$-AR signaling to ACs to produce cAMP, we first investigated the role of $G\alpha_s$ in this biphasic dose–response. In MEF cells, isoproterenol increased the cellular cAMP levels with an $EC_{50}$ of $\sim 30$ nM (Figure 1E). This $EC_{50}$ value is closer to the $EC_{50}$ of the first phase of the biphasic dose–response, implying that $G\alpha_s$ might be responsible for the first response phase. To examine the role of $G\alpha_s$, we used a genetic approach by studying isoproterenol stimulation of ERK MAPK activity in $G\alpha_s^{-/-}$ MEF cells (Bastepe *et al*, 2002). In $G\alpha_s^{-/-}$ cells, isoproterenol was unable to increase the cellular cAMP levels, confirming the functional absence of $G\alpha_s$ (Figure 1F). On the other hand, forskolin (directly activating ACs) increased cAMP in $G\alpha_s^{-/-}$ cells (Figure 1G). When ERK MAPK activity was examined, the first phase of the dose–response curve of isoproterenol stimulation of ERK MAPK was absent whereas the second phase was almost intact with an $EC_{50}$ of 10 μM (Figure 1H and I). From radio-ligand binding experiments, $G\alpha_s^{-/-}$ cells had a similar $\beta_2$-AR density on the membrane as wild-type MEFs. $G\alpha_s^{-/-}$ cells possess $0.40 \pm 0.07$ pmol/mg of membrane protein ($n = 3$), whereas wild-type MEF cells have $0.55 \pm 0.11$ pmol/mg of membrane protein ($n = 3$). These molecular genetic data clearly demonstrate that $G\alpha_s$ is responsible for the first phase of the response corresponding to low isoproterenol dosages, and that $G\alpha_s$ is not essential for the second phase of the response corresponding to high isoproterenol dosages, implying that the second phase could be through another pathway that is $G\alpha_s$ independent.

### *The second phase of the response requires β-ARs*

We then focused on the signaling pathway that is responsible for the second phase of the dose–response curve. First, we asked whether this response is still through $\beta_2$-AR. In $\beta_1$-$AR^{-/-}\beta_2$-$AR^{-/-}$ MEF cells ($\beta_1^{-/-}\beta_2^{-/-}$ cells), isoproterenol, at low and high concentrations, was unable to increase cellular cAMP levels (Figure 2A). However, $\beta_1^{-/-}\beta_2^{-/-}$ cells gave rise to increased cAMP levels in response to forskolin (Figure 2B). Furthermore, in $\beta_1^{-/-}\beta_2^{-/-}$ cells, isoproterenol at low and high concentrations did not activate ERK MAPK (Figure 2C). These data demonstrate that isoproterenol at the concentrations that we used here still acts through β-ARs. These data validate that the second phase of the response is still a β-AR-mediated event.

### *Neither $G\alpha_i$ nor β-arrestins mediate the second phase of the response*

Next, we examined the signaling molecules downstream of $\beta_2$-AR that are directly responsible for the second phase of the dose–response curve. In *in vitro* reconstitution studies, it was shown that $\beta_2$-AR was also capable of coupling to $G\alpha_i$ (Cerione *et al*, 1985; Rubenstein *et al*, 1991). To study a possible role of $G\alpha_i$ in the second phase, we treated $G\alpha_s^{-/-}$ cells with PTX, a toxin that would inhibit $G\alpha_i$, and then measured the dose–response of isoproterenol stimulation of ERK MAPK. As shown in Figure 2D and E, PTX treatment had no effect on the $EC_{50}$ ($\sim 10$ μM) of $G\alpha_s^{-/-}$ cells. Under similar experimental conditions, PTX blocked the stimulation of ERK MAPK by Gi-coupled m2 muscarinic acetylcholine receptors (Figure 2F). Furthermore, expression of RGS4 (a GAP for $G\alpha_q$ and $G\alpha_i$ proteins) and the RGS-like domain of p115 Rho-GEF (a GAP for $G\alpha_{12}$ and $G\alpha_{13}$ proteins) in $G\alpha_s^{-/-}$ cells did not affect the ERK activation by 10 μM of isoproterenol (Figure 2G). These data rule out a primary role for heterotrimeric G proteins in the second phase of ERK MAPK activation by isoproterenol.

It has been postulated that $\beta_2$-AR, after internalization, could initiate a second wave of signaling events including the activation of ERK MAPK (Luttrell *et al*, 1999). Therefore, we examined whether $\beta_2$-AR internalization is responsible for the second phase. β-Arrestin 2 has been shown to be absolutely required for $\beta_2$-AR internalization in MEF cells (Kohout *et al*, 2001; Huang *et al*, 2004). Indeed, in β-arrestin $2^{-/-}$ cells and β-arrestin $1^{-/-}$/β-arrestin $2^{-/-}$ cells, we observed that $\beta_2$-AR internalization was blocked (Huang *et al*, 2004). However, β-arrestin $2^{-/-}$ cells and β-arrestin $1^{-/-}$/β-arrestin $2^{-/-}$ cells displayed a similar biphasic dose–response curve of isoproterenol stimulation of ERK MAPK to that in wild-type MEF cells (Figure 2H–K). This genetic evidence demonstrates that $\beta_2$-AR internalization is not responsible for the second phase.

### *Src tyrosine kinase is required for the second phase of the response*

Src tyrosine kinase has been shown to mediate $\beta_2$-AR cellular signaling at several levels (Luttrell *et al*, 1999; Ma *et al*, 2000; Schmitt and Stork, 2002b). Therefore, we turned our attention to Src for a possible role in transducing the second phase of the isoproterenol stimulation of ERK MAPK. In Src-family tyrosine kinase-deficient SYF cells, low concentrations of isoproterenol increased the ERK MAPK activity with an $EC_{50}$ of $\sim 30$ nM (Figure 3A and B). (SYF cells are MEF cells derived from $Src^{-/-}Yes^{-/-}Fyn^{-/-}$ mouse embryos.) Remarkably, high concentrations of isoproterenol did not further increase the ERK MAPK activity (Figure 3A and B). This defect was due to the absence of Src-family tyrosine kinases as re-expression of c-Src in SYF cells restored the biphasic response of ERK activation by isoproterenol (Figure 3C and D). The $EC_{50}$ for isoproterenol increasing cellular cAMP in SYF cells was also $\sim 30$ nM (Figure 3E), a value that is same as that in wild-type MEF cells. Furthermore, the ERK activation in $G\alpha_s^{-/-}$ cells by 100 μM isoproterenol was abolished by 10 μM PP2, an Src-family tyrosine kinase inhibitor (Figure 1I). Moreover, from radio-ligand binding experiments, SYF cells had a similar $\beta_2$-AR density on the membrane as wild-type MEFs. SYF cells possess $0.44 \pm 0.09$ pmol/mg of membrane protein ($n = 3$), whereas wild-type MEF

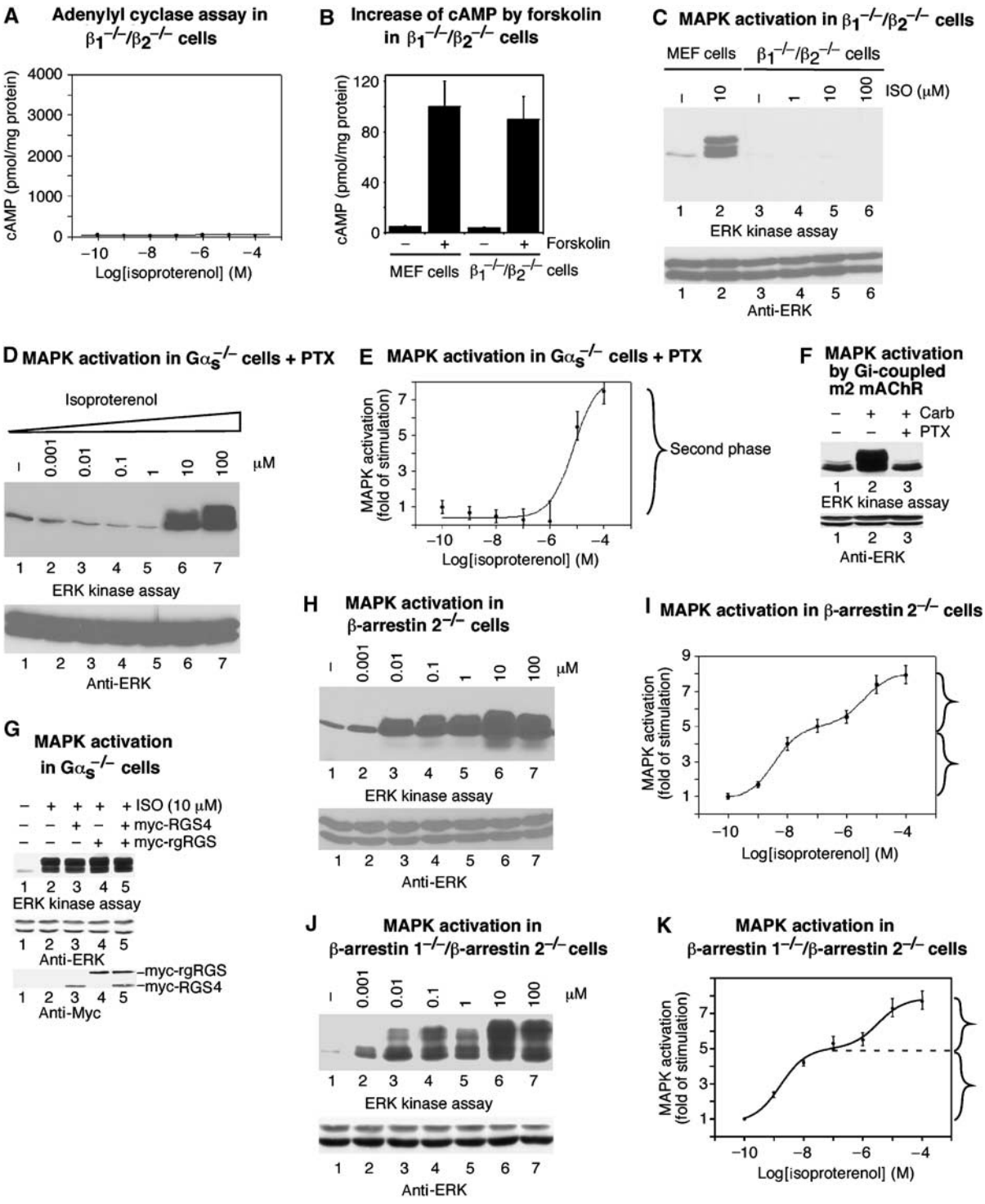


**Figure 2** The second phase of the stimulation of ERK MAPK by $\beta_2$-AR does not require G proteins and receptor internalization. (**A**) No stimulation of ACs by isoproterenol in $\beta_1$-AR$^{-/-}$/$\beta_2$-AR$^{-/-}$ cells. (**B**) Increase of cAMP by forskolin in $\beta_1$-AR$^{-/-}$/$\beta_2$-AR$^{-/-}$ cells. (**C**) No stimulation of ERK MAPK by isoproterenol in $\beta_1$-AR$^{-/-}$/$\beta_2$-AR$^{-/-}$ cells. Stimulation of ERK MAPK in MEF cells was used as positive control (lanes 1 and 2). (**D, E**) PTX pre-treatment did not alter the dose–response curve of ERK MAPK stimulation by isoproterenol in G$\alpha_s^{-/-}$ cells. Data represent mean$\pm$s.d. of three experiments. (**F**) PTX pre-treatment blocked the ERK MAPK stimulation by Gi-coupled m2 mAChR in G$\alpha_s^{-/-}$ cells. (**G**) Expression of RGS4 and the RGS domain of p115 Rho GEF had no effect on ERK MAPK stimulation by isoproterenol in G$\alpha_s^{-/-}$ cells. (**H, I**) Different concentrations of isoproterenol increased the kinase activity of ERK MAPK in β-arrestin 2$^{-/-}$ cells. (**J, K**) Different concentrations of isoproterenol increased the kinase activity of ERK MAPK in β-arrestin 1$^{-/-}$/β-arrestin 2$^{-/-}$ cells. Data represent mean$\pm$s.d. of three experiments.

cells have $0.55\pm0.11$ pmol/mg of membrane protein ($n=3$). These genetic data demonstrate that, in SYF cells, G$\alpha_s$-mediated $\beta_2$-AR signaling is intact whereas the second phase of the ERK MAPK activation was abolished. Thus, Src-family tyrosine kinases are required for the second phase of the response.

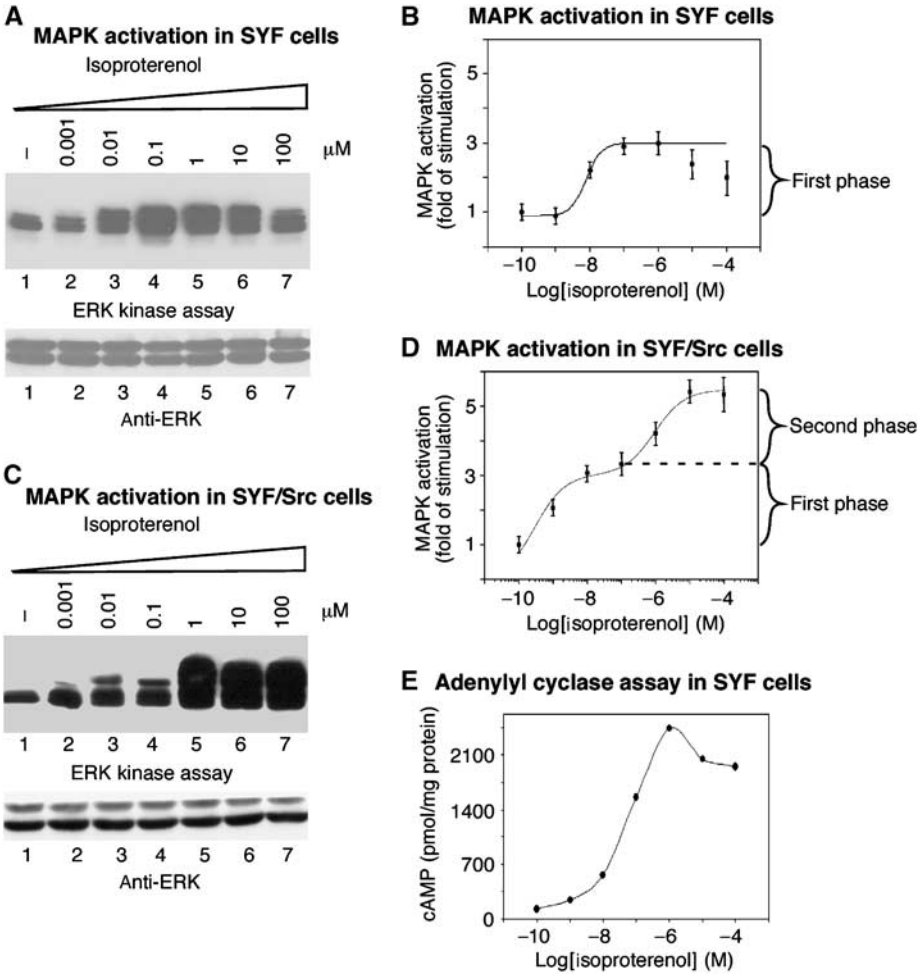


**Figure 3** Src is required for the second phase of the stimulation of ERK MAPK by $\beta_2$-AR. (**A, B**) Effect of different concentrations of isoproterenol on the kinase activity of ERK MAPK in SYF cells. (**C, D**) Effect of different concentrations of isoproterenol on the kinase activity of ERK MAPK in SYF/c-Src cells. Data represent mean $\pm$ s.d. of three experiments. (**E**) Dose–response of cAMP production by isoproterenol stimulation in SYF cells.

### *Direct interaction of $\beta_2$-AR with Src*

Having established that Src-family tyrosine kinases are required for the second phase of the isoproterenol stimulation of ERK MAPK, we then investigated the biochemical mechanism by which $\beta_2$-AR is connected to Src. Src has been shown to be activated by $\beta_2$-AR signals through several mechanisms: by direct $G\alpha_s$ contact (Ma *et al*, 2000), by PKA phosphorylation (Schmitt and Stork, 2002b), and by β-arrestin recruitment (Luttrell *et al*, 1999). As $G\alpha_s$ and β-arrestin 2 are not essential for the second phase of ERK MAPK activation, there might be an additional route(s) for $\beta_2$-AR activation of Src. We found that, in $G\alpha_s^{-/-}$ cells and β-arrestin $1^{-/-}$/β-arrestin $2^{-/-}$ cells, isoproterenol (10 μM) was still able to stimulate Src (Figure 4A). This finding indeed suggested that another Src activation pathway is employed by $\beta_2$-AR in transducing the isoproterenol stimulation in the second phase of the response.

Next, we performed biochemical studies with purified Src tyrosine kinase and the purified C-terminal tail (amino-acid residues 331–413) of $\beta_2$-AR to examine whether $\beta_2$-AR could directly interact with Src, and whether Src could directly phosphorylate $\beta_2$-AR. First, we found that c-Src could directly interact with the C-terminal tail of $\beta_2$-AR, and that this binding depends on the phosphorylation states of both proteins. When the C-terminal fragment of $\beta_2$-AR (as a GST fusion protein) was unphosphorylated, purified unphosphorylated Src could bind to it (Figure 4B). (The purity of these recombinant proteins is shown in the bottom panel of Figure 4E.) Pre-activated Src (pre-incubation with ATP to induce autophosphorylation at Tyr-416 of the activation loop of c-Src) or Csk-inactivated Src (Tyr-527 phosphorylated by Csk) did not bind to the unphosphorylated C-terminal tail of $\beta_2$-AR (Figure 4C, lanes 3 and 4, and data not shown). On the other hand, pre-activated Src bound to the phosphorylated C-terminal tail of $\beta_2$-AR (Figure 4C, lanes 1 and 2). Furthermore, endogenous c-Src could be co-immunoprecipitated with endogenous $\beta_2$-AR in cells with or without isoproterenol stimulation, confirming a previous report (Fan *et al*, 2001) (Figure 4D). Second, we examined whether Src could directly phosphorylate the C-terminal tail of $\beta_2$-AR. As shown in Figure 4E, purified Src was able to phosphorylate the purified C-terminal tail of $\beta_2$-AR. Expression of a constitutively active Src in cells also led to increased phosphorylation of $\beta_2$-AR (data not shown). Together, these data demonstrate that $\beta_2$-AR could directly interact with Src.

### *$\beta_2$-AR could directly activate Src*

Thinking of the mode of direct activation of JAK tyrosine kinases by cytokine receptors, we investigated the possibility

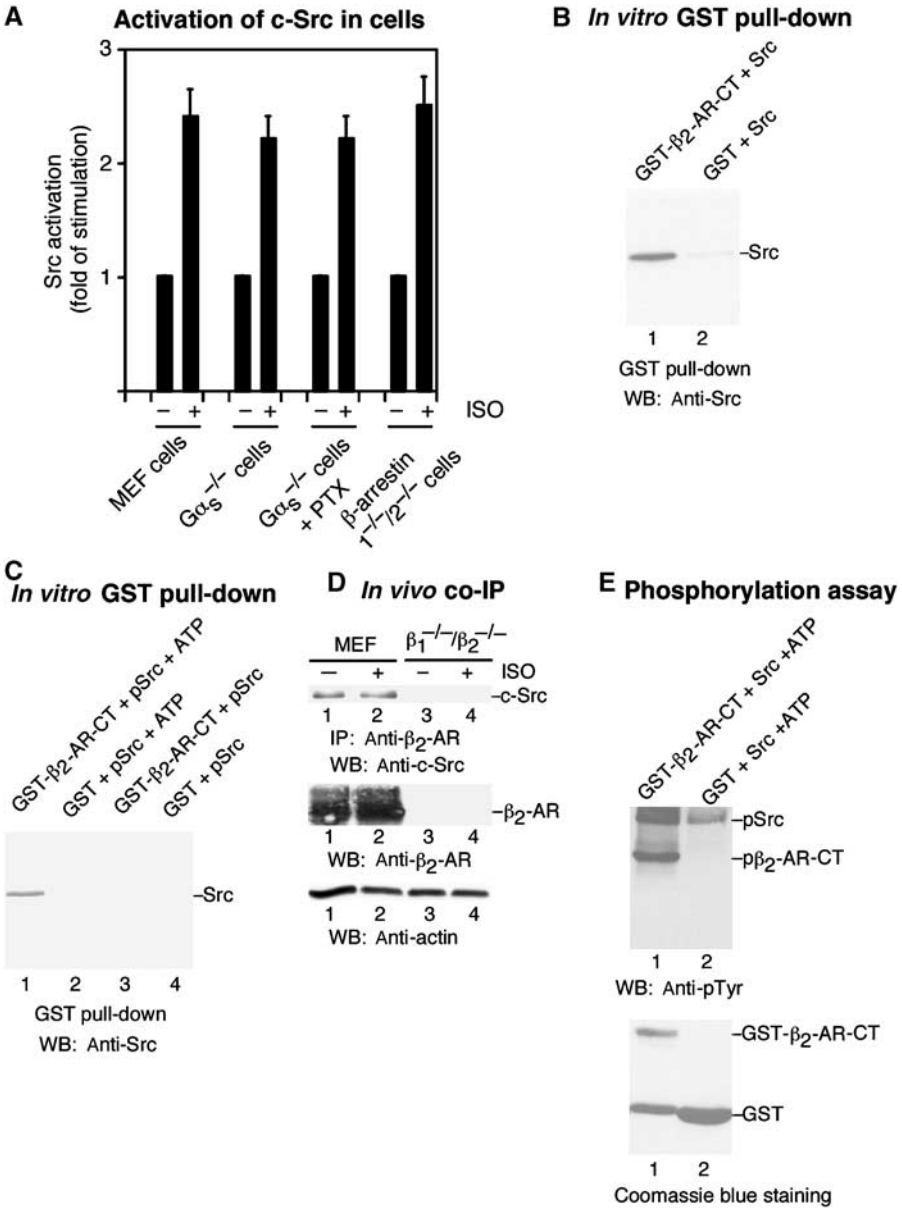


**Figure 4** Direct interaction between Src proteins and $\beta_2$-AR. (**A**) Stimulation of c-Src kinase activity by 10 μM isoproterenol in MEF, $G\alpha_s^{-/-}$, and β-arrestin $1^{-/-}2^{-/-}$ cells. Data represent mean ± s.d. of three experiments. (**B**) *In vitro* binding assays of purified unphosphorylated Src with the purified unphosphorylated C-terminal tail of $\beta_2$-AR (as GST fusion protein). GST alone was used as a negative control. (**C**) Purified phosphorylated Src was assayed for interaction with the C-terminal tail of $\beta_2$-AR in the presence or absence of ATP. (**D**) Co-immunoprecipitation of endogenous $\beta_2$-AR with endogenous c-Src from HEK-293 cells in the presence or absence of isoproterenol. (**E**) Phosphorylation of the purified C-terminal tail of $\beta_2$-AR by purified Src. Data are representative of three experiments.

of direct activation of Src by $\beta_2$-AR as $\beta_2$-AR could directly bind to Src. We purified recombinant human $\beta_2$-AR, c-Src, and $G\beta_1\gamma_2$ proteins from Sf9 cells, and recombinant $G\alpha_s$ proteins from *Escherichia coli* (Figure 5A). When Src is activated, it can autophosphorylate the Tyr-416 residue. We used this increase of tyrosine autophosphorylation as a measure for Src activation. As shown in Figure 5B and C, in the absence of ATP, Src was not tyrosine phosphorylated (Figure 5B, lane 1). Addition of ATP led to basal Src autophosphorylation (Figure 5B, lane 2). In the absence of agonist or in the presence of an antagonist, $\beta_2$-AR did not increase this basal autophosphorylation (Figure 5B, lanes 3 and 4). Significantly, when purified $\beta_2$-AR was reconstituted with purified Src, addition of isoproterenol increased the extent of tyrosine phosphorylation of Src (Figure 5B, lane 5). This increase of Src activity by isoproterenol was blocked by pretreatment with the $\beta_2$-AR antagonist alprenolol (Figure 5B, lane 6). Furthermore, using GST-CDB3 fusion protein as an exogenous substrate for Src, similar results were obtained (Figure 5D and E). These data demonstrate that $\beta_2$-AR could directly activate Src and that Src activation by $\beta_2$-AR is agonist-dependent.

As GPCRs activate G proteins by accelerating the rate of guanine nucleotide exchange, we further studied the biochemical mechanism by which $\beta_2$-AR activates Src by examining the rate of Src activation by $\beta_2$-AR. We first tested our purified $\beta_2$-AR proteins on Gs activation (Figure 5F). In the presence of agonist isoproterenol, GTPγS binding to Gs

 

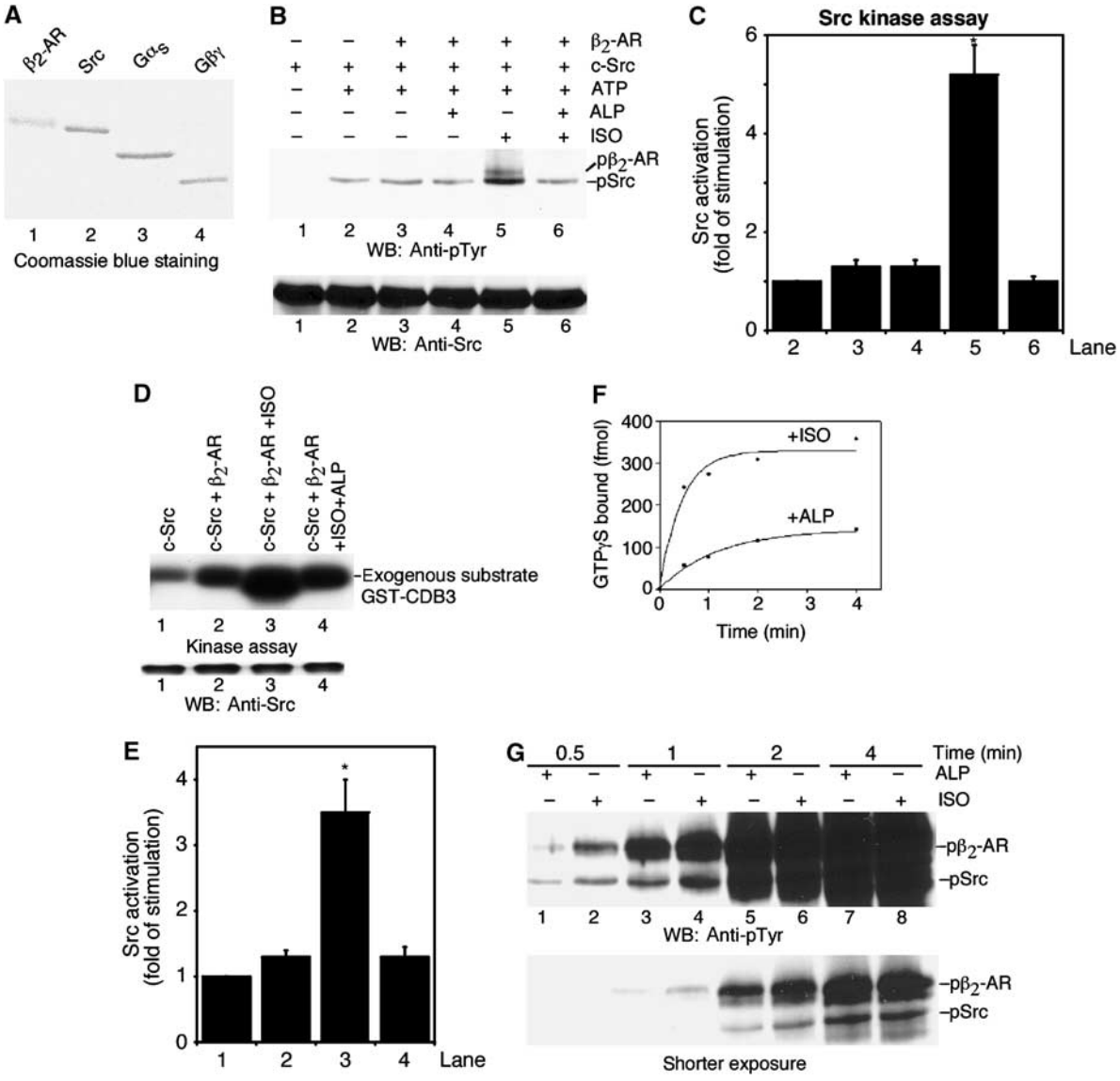


**Figure 5** Direct activation of Src by $\beta_2$-AR. (**A**) Coomassie blue staining of purified human $\beta_2$-AR from Sf9 cells, purified c-Src from Sf9 cells, purified $G\alpha_s$ from *E. coli*, and purified $G\beta_1\gamma_2$ from Sf9 cells ($G\gamma_2$ protein was off the gel). (**B**) Purified $\beta_2$-AR directly activated purified c-Src. Top panel: Western blot with anti-phospho-tyrosine antibody to show the autophosphorylation of Src (pSrc) and the phosphorylation of $\beta_2$-AR by Src ($p\beta_2$-AR) (the reaction was for ~30 s) (10% SDS–PAGE). ALP: alprenolol; ISO: isoproterenol. Bottom panel: the same filter was probed with anti-Src antibody to show that similar amounts of Src were used in each reaction. (**C**) Quantification of data in (B). Error bars show mean ± s.d., $^*P<0.001$ (Student's *t*-test). (**D**) Purified $\beta_2$-AR directly activated purified c-Src. GST-CDB3 fusion protein was used as exogenous substrate for Src. (**E**) Quantification of data in (D). Error bars show mean ± s.d., $^*P<0.001$ (Student's *t*-test). (**F**) Acceleration of GTPγS binding to Gs ($\alpha_s+\beta\gamma$) by $\beta_2$-AR. $G\alpha_s$, $G\beta\gamma$, and $\beta_2$-AR together with alprenolol (■) or isoproterenol (●) were incubated on ice for 10 min. After incubation at 30°C for 5 min, [$^{35}$S]GTPγS was added. At various time points, aliquots were removed and $^{35}$S was counted to measure GTPγS loading. (**G**) $\beta_2$-AR increased the autophosphorylation of Src (as well as the phosphorylation of $\beta_2$-AR) after incubations for 30 s, 1 min, and 2 min (7% SDS–PAGE). After 4 min incubation, there was no difference between the phosphorylation with or without ISO. Bottom panel: a shorter ECL exposure of the same filter shown above. Data are representative of three to five experiments.

($\alpha_s+\beta\gamma$) was faster than in the presence of antagonist alprenolol (Figure 5F). The reason for adding alprenolol was to reduce the basal activity of purified $\beta_2$-ARs. Then we examined the time course of Src activation by $\beta_2$-AR. Similar to the activation of G proteins, $\beta_2$-AR accelerated the rate of Src activation. In the short time points (0.5, 1, and 2 min), the degree of Src autophosphorylation in the presence of isoproterenol was higher than that in the presence of alprenolol (Figure 5G, lanes 1–6). However, after 4-min incubation, Src autophosphorylation with isoproterenol or alprenolol showed similar levels (Figure 5G, bottom panel, lanes 7 and 8). Hence, $\beta_2$-AR increases the rate of Src activation, similar to G-protein activation.

To further link the Src and $\beta_2$-AR interaction to the second phase of the ERK activation by $\beta_2$-AR, we have performed mutagenesis studies. We wanted to identify a $\beta_2$-AR-mutant that would not bind to c-Src and then to examine whether $\beta_1^{-/-}/\beta_2^{-/-}$ cells expressing this $\beta_2$-AR-mutant would lack the second phase of the ERK response. As the C-terminal tail (residues 331–413) of $\beta_2$-AR was sufficient for binding to c-Src and the C-terminal tail of turkey β-AR could not bind to c-Src (Figure 6A and B), we have made several chimeras of

these C-terminal tails to change some of the $\beta_2$-AR residues to those in turkey β-AR. Among these chimeras, a $\beta_2$-AR-mutant (named $\beta_2$-AR-mutant-2 in Figure 6A) was unable to bind to c-Src (Figure 6B). This $\beta_2$-AR-mutant contains three amino-acid changes in the proposed helix 8 (based on the crystal structure of rhodopsin) (Palczewski *et al*, 2000). As a control, an adjacent mutation (named $\beta_2$-AR-mutant-1 in Figure 6A) was used and shown to bind to c-Src. These $\beta_2$-AR-mutants were made on the GST-$\beta_2$-AR C-terminal tail (residues 331–413) backbone. Both these $\beta_2$-AR-mutants had no defects in activating Gs, as measured by the cAMP assay in response to isoproterenol (Figure 6C). We then established $\beta_1^{-/-}/\beta_2^{-/-}$ cells stably expressing the $\beta_2$-AR-mutant-2. In these cells, the first phase of the ERK response after isoproterenol stimulation was intact whereas the second phase of the response was absent (Figure 6D and E). These data show that a $\beta_2$-AR-mutant defective in c-Src binding leads to the absence of the second phase of the ERK response, consistent with a role for c-Src in the second phase of the activation of ERK by $\beta_2$-AR.

## Discussion

Our new finding of the dose-dependent shift of signaling modes by a GPCR has several implications. G-protein-dependent signaling and G-protein-independent signaling are related by their different sensitivity to concentrations of the same agonist. The G-protein-dependent signaling of GPCRs is similar to receptor tyrosine kinase signaling through the small GTPase Ras. The G protein-independent signaling of GPCRs can be compared to cytokine JAK–STAT signaling, in which non-receptor tyrosine kinases are directly coupled to membrane receptors. Also, it is interesting to note that some seven-transmembrane receptors, such as Smoothened in Hedgehog gradient signaling, mainly use their C-terminal

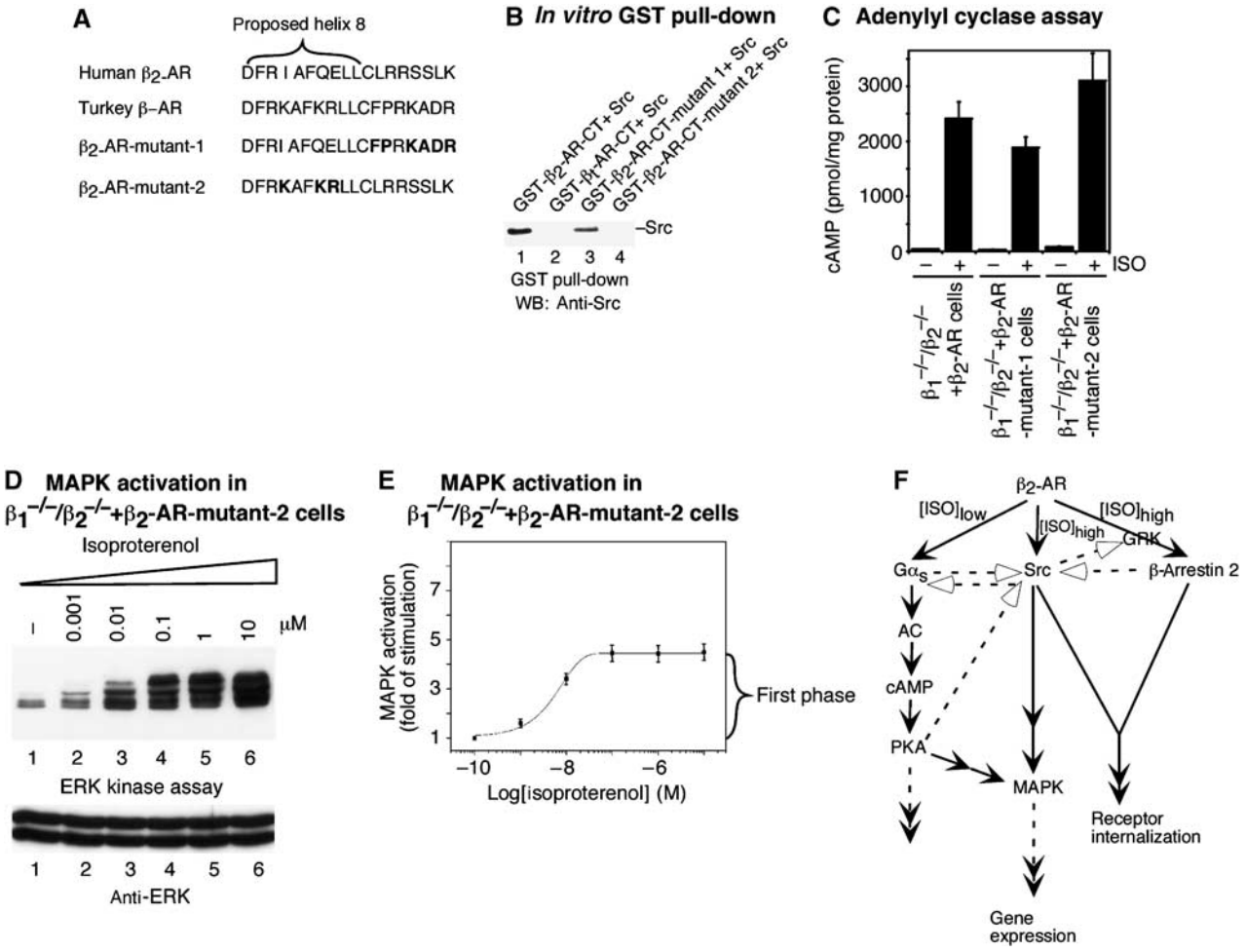


**Figure 6** A $\beta_2$-AR-mutant defective in c-Src binding abolishes the second phase of the ERK response. (**A**) Sequence alignment of the relevant regions of the $\beta_2$-AR and turkey β-AR (the sequences of the remaining C-terminal tails are not shown). (**B**) *In vitro* GST-pull-down assay with purified GST fusion proteins and purified c-Src. (**C**) cAMP assays with $\beta_1^{-/-}/\beta_2^{-/-}$ cells and $\beta_1^{-/-}/\beta_2^{-/-}$ cells expressing the $\beta_2$-AR-mutants. (**D, E**) Different concentrations of isoproterenol increased the kinase activity of ERK MAPK in $\beta_1^{-/-}/\beta_2^{-/-}$ cells expressing the $\beta_2$-AR-mutant-2. (**F**) Diagram of G-protein-dependent and -independent pathways initiated from $\beta_2$-AR. At low concentrations of isoproterenol, $\beta_2$-AR signals through $G\alpha_s$ to activate ACs to produce cAMP. At high concentrations of isoproterenol, $\beta_2$-AR could directly activate c-Src leading to the activation of ERK MAPK. Also, at high concentrations of isoproterenol, $\beta_2$-AR initiates its own internalization. Both Src and β-arrestin 2 are required for receptor internalization. There is quite an amount of crosstalk in these pathways (indicated by open arrows) such as direct activation of Src by $G\alpha_s$, by PKA phosphorylation, or by β-arrestin recruitment. Src could also phosphorylate $G\alpha_s$ and enhance the activity of $G\alpha_s$. Src has also been reported to phosphorylate and activate GRKs. This extensive crosstalk might underline the shift of $EC_{50}$ values in $G\alpha_s^{-/-}$ and SYF cells compared to wild-type MEF cells. cAMP and PKA could directly act on target proteins to induce immediate responses. The ERK MAPK pathway could work through gene expression to induce a long-lasting effect.

tails to directly interact with downstream signaling molecules without the participation of G proteins (Lum and Beachy, 2004). Both the JAK–STAT and the Smoothened pathways result in activation of latent cytoplasmic transcription factors. Src could mediate GPCR-responsive changes in gene expression (Figure 6F). Furthermore, it is known that high-affinity agonist binding of GPCRs requires a functional interaction between the receptors and G proteins. This behavior is consistent with our observation that the G-protein-coupled signaling requires low concentrations of agonists whereas non-G-protein signaling needs high concentrations of agonists. We should point out that the maximum ERK MAPK responses at high concentrations of ligands are contributed by both $G\alpha_s$-mediated and Src-mediated pathways and result in the summation of responses from both pathways. The relative contributions of the first phase and the second phase to the maximum response are approximately the same. One explanation for this observation is that $\beta_2$-AR, at high concentrations, exists in two distinct conformations. Approximately half of the $\beta_2$-AR population is in a conformation that is compatible with G-protein coupling, whereas the other half is in a conformation that is not compatible with G-protein-coupling. Rather, these receptors could interact with and activate Src. In this regard, it is interesting to note that purified $\beta_2$-ARs, in the presence of high concentrations of agonist (100 μM of isoproterenol), have been shown to exist in two distinct biophysical conformations, suggesting that there is more than one active biophysical conformation for a given receptor (Swaminath *et al*, 2004). We speculate that these two structurally different conformations might correspond to the two functionally different conformations that we propose here. Thus, it is possible that one agonist could induce discrete receptor conformations permitting the receptor to recognize different interacting effectors (e.g., $G\alpha_s$ or Src) and to activate the same or different cellular pathways. Future structural and functional (G-protein coupling versus Src coupling) investigations should shed light on this fundamental receptor property.

We propose a model for the direct activation of Src by $\beta_2$-AR. The mechanism is similar to that of the direct activation of tyrosine kinase JAK by cytokine receptors. In this model, unphosphorylated Src (partially active) is associated with the C-terminal tail of unphosphorylated $\beta_2$-AR in the absence of ligands. As Csk-phosphorylated c-Src could not bind to the C-terminal tail of $\beta_2$-AR, the c-Src proteins that are constitutively associated with $\beta_2$-ARs are likely dephosphorylated at the C-terminal tail tyrosine residue before ligand stimulation. Ligand binding of $\beta_2$-AR leads to the activation of Src. It is speculated that high concentrations of ligands could induce $\beta_2$-AR dimerization or cause conformational changes (or stabilization) of pre-formed $\beta_2$-AR dimers. Although we have not examined dimerization here, there have been numerous reports of GPCR dimerization including $\beta_2$-AR (Bouvier, 2001). This dimerization or the conformational change (more likely) would bring two Src molecules to proximity and lead to intermolecular autophosphorylation at Tyr-416 and full activation. Activated Src could signal to downstream targets, such as the ERK MAPK pathway. Furthermore, activated Src could phosphorylate the C-terminal tail of $\beta_2$-AR (activated Src could temporarily dissociate from, phosphorylate, and rebind $\beta_2$-AR). These phosphorylated tyrosine residues on $\beta_2$-AR could then serve as potential docking sites for other signaling molecules. Receptor-bound activated Src could also phosphorylate other proteins. These would lead to $G\alpha_s$-independent and Src-dependent signaling pathways, contributing to the increase in ERK MAPK activity in the second phase and other physiological responses.

A Gi protein-dependent and agonist-induced co-immunoprecipitation of activated c-Src with $\beta_3$-AR was reported previously (Cao *et al*, 2000). $\beta_3$-AR utilized a PTX-sensitive intracellular pathway to activate c-Src. This activated c-Src was recruited to $\beta_3$-AR by interacting with the proline-rich motifs in the third intracellular loop and the C-terminal tail of $\beta_3$-AR. It was shown that the peptides from the third intracellular loop or from the C-terminal tail of $\beta_3$-AR could bind to GST-Src SH3/SH2 fusion protein, but not to GST-Src SH2 fusion protein. There are several differences in the $\beta_3$-AR and c-Src interaction and the interaction between $\beta_2$-AR and c-Src reported here. First, the $\beta_2$-AR and c-Src interaction was G-protein-independent whereas the $\beta_3$-AR and c-Src interaction was Gi-dependent. Second, $\beta_2$-AR directly activated c-Src *in vitro*, in the absence of G proteins. $\beta_3$-AR did not directly activate c-Src, instead utilized a Gi-dependent intracellular pathway to activate c-Src. Third, $\beta_2$-AR and c-Src interaction was constitutive whereas $\beta_3$-AR and c-Src interaction was agonist-dependent. Fourth, both inactive and active c-Src interacted with $\beta_2$-AR whereas only activated c-Src interacted with $\beta_3$-AR.

Our finding has a significant implication for receptor desensitization. Receptor desensitization reflects no further increase or even a decline of a physiological response in the continuous presence of ligand. Depending on the physiological responses (or readouts), the underlying biophysical basis could be different. If GPCR desensitization is defined as its uncoupling from G proteins, our data of the shift of signaling modes by increasing ligand concentrations suggest that GPCR desensitization (excluding receptor internalization and downregulation) is an intrinsic property of the receptor proteins induced by ligand binding, similar to the inactivation property of ion channel proteins. Although no auxiliary protein factors (such as GRK and arrestin proteins) are essential for this G-protein decoupling, these proteins could directly or indirectly facilitate and amplify the desensitization processes in cells (such as through receptor internalization and downregulation).

## Materials and methods

### *Cell lines*

The MEF cells deficient of $G\alpha_s$ ($G\alpha_s^{-/-}$ cells) were derived from $G\alpha_s$ (exon 2) knockout mice (Bastepe *et al*, 2002). The MEF cells deficient of both $\beta_1$-AR and $\beta_2$-AR ($\beta_1^{-/-}\beta_2^{-/-}$ cells) were derived from $\beta_1$-AR and $\beta_2$-AR double knockout mouse embryos (Rohrer *et al*, 1999). The MEF cells deficient of Src-family tyrosine kinases (SYF cells) were purchased from ATCC. The MEF cells deficient of β-arrestin 1 and 2 were kindly provided by Dr R Lefkowitz (Duke University) (Kohout *et al*, 2001).

### *cAMP assay*

The cAMP assay was performed as described previously (Huang *et al*, 2004). Cells were plated onto six-well plates and treated with 1 mM IBMX for 30 min. After washing twice with HEM buffer (20 mM Hepes, pH 7.4, 135 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $NaHCO_3$, 0.1 mM Ro-20-1724, 0.5 U/ml adenosine deaminase, and 1 mM IBMX), cells were treated with isoproterenol in HEM buffer for 5 min. After two more washes with HEM buffer, cells were harvested in 0.5% Triton X-100 containing 1 mM IBMX. The amount of cAMP was measured with the Direct Cyclic AMP Enzyme Immunoassay kit (Assay Designs Inc.).

### *ERK MAPK assay*

The p44/42 MAP kinase assay was performed using a kit from Cell Signaling Technology as described previously (Wan *et al*, 1996). Whole-cell lysates were prepared from MEF, $G\alpha_s^{-/-}$, $\beta_1^{-/-}\beta_2^{-/-}$, SYF, β-arrestin $2^{-/-}$, or β-arrestin $1^{-/-}2^{-/-}$ fibroblast cells. Cells were either treated or not with isoproterenol for 5 min. A monoclonal antibody to the phospho-p44/42 ERK MAPK (crosslinked to agarose beads) was added to immunoprecipitate the active ERK MAPK from cell lysates. Substrates (200 μM ATP and 2 μg GST-Elk-1 fusion protein) were added and the reaction was allowed to proceed at 30°C for 30 min. After SDS–PAGE, the ERK MAPK activity (the phosphorylation of GST-Elk-1 by ERK MAPK) was measured by Western blotting with an anti-phospho-Elk-1 antibody (Cell Signaling Technology). In some experiments, cells were treated with 100 ng/ml PTX overnight. The intensity of the bands on the films from ERK kinase assays was scanned with a densitometer (KODAK). The data fitting was performed with GraphPad Prism.

### *Radio-ligand receptor binding assay*

The radio-ligand binding assay was performed as described previously (January *et al*, 1997). Membrane preparations were incubated with [$^3$H]CGP-12177 (100 nM) in the absence and presence of 1 mM terbutaline (to define nonspecific binding) in 25 mM Tris–HCl buffer (pH 7.4 at 37°C) containing 154 mM NaCl. Incubation was carried out at 37°C for 120 min, which was found to be optimal for specific binding. Incubations were performed in triplicate. The incubation was terminated by rapid vacuum filtration through GF/C glass-fiber filters. Each filter was rapidly washed with $3 \times 5$ ml ice-cold 25 mM Tris–HCl buffer (pH 7.4). The filters were counted.

### *Cellular Src activation assay*

The *in vivo* Src activation assay was performed as described previously (Wan *et al*, 1996). MEF, $G\alpha_s^{-/-}$, and β-arrestin $1^{-/-}2^{-/-}$ cells were challenged with or without 10 μM isoproterenol for 5 min after serum starvation for 16 h. Cells were harvested in lysis buffer (40 mM Hepes, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% dodecyl-β-D-maltoside (DoDM), 2 mM $Na_3VO_4$, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.2 mM PMSF). One microgram of anti-c-Src (B12 from Santa Cruz) antibody together with cell lysate containing 1.8 mg protein in 1 ml was incubated at 4°C for 2 h before 20 μl of protein A/G agarose beads was added. After overnight incubation at 4°C, the beads were spun down and washed twice with lysis buffer. After two more washes with kinase buffer (40 mM Hepes, pH 7.5, 5 mM $MnCl_2$, 1 mM $MgCl_2$, and 1 mM DTT), the beads were mixed with 45 μl kinase buffer containing 5 μg GST-CDB3 and 5 μCi [γ-$^{32}$P]ATP and incubated at 30°C for 30 min. The reaction was terminated by adding SDS loading buffer. After incubation at 95°C for 5 min, GST-CDB3 was separated on a 7% SDS–PAGE gel, dried, and autoradiographed. In some experiments, cells were treated with 100 ng/ml PTX overnight before isoproterenol stimulation.

### *In vitro binding assay*

The *in vitro* binding assay was performed as described previously (Ma and Huang, 1998; Lowry *et al*, 2002). Eight micrograms of GST or GST fusion proteins and 2.5 μg c-Src together with 20 μl 50% glutathione agarose were incubated in 0.5 ml ice-cold binding buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mg/ml BSA, and 0.1% NP-40) at 4°C overnight. For phosphorylation-dependent bindings, 8 μg GST or GST fusion proteins and 2.5 μg c-Src together with 1 mM ATP were incubated in 50 μl kinase buffer (20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$) at 30°C for 30 min. The reaction was stopped by adding 450 μl ice-cold binding buffer and 20 μl 50% glutathione agarose and incubated at 4°C overnight. The beads were washed three times with 1 ml washing buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, and 0.1% NP-40). The protein complex was eluted with elution buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10 mM glutathione, and 0.1% NP-40) and detected by anti-c-Src antibody (B12, Santa Cruz Biotechnology).

### *Protein purification*

Human $\beta_2$-AR protein was purified as described previously (Kobilka, 1995). Purification of c-Src proteins was described before (Ma *et al*, 2000). Purification of $G\alpha_s$ protein was as previously described with some modifications (Ma *et al*, 2000). Briefly, pGEX 6P-$G\alpha_s$ plasmid was transformed into the bacterial strain BL21(DE3). One liter of bacterial culture was grown at room temperature until the absorbance at 600 nm was ~1. $G\alpha_s$ protein expression was induced with 0.5 mM IPTG for 18 h at room temperature. The bacterial pellet was resuspended in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1 mg/ml lysozyme, and 0.2 mM PMSF) and incubated on ice for 30 min. After sonication, the lysate was spun down at 10 000 *g* for 2 h at 4°C. Glutathione agarose resin (0.5 ml, from Sigma) was added to the supernatant after pre-equilibration of the resin with lysis buffer. The mixture was gently agitated at 4°C for 3 h. After washing three times with 10 ml washing buffer (50 mM Tris, pH 8.0, 100 mM NaCl, and 0.2 mM PMSF), GST-tagged $G\alpha_s$ was eluted with 0.5 ml elution buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 10% glycerol). preScission protease (Amersham Biosciences) was used to cleave GST at 4°C overnight.

Purification of Gβγ proteins was as previously described with some modifications (Lowry *et al*, 2002). After infection (for 72 h) with recombinant baculoviruses encoding $G\beta_1$ and $G\gamma_2$, the pellet from 1 l Hi5 cells was resuspended in 50 ml lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, and protease inhibitors: 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM benzamidine, and 0.2 mM PMSF). After sonication, the lysate was spun down at 150 000 *g* for 90 min at 4°C. The membrane pellet was homogenized in 50 ml lysis buffer. After centrifugation, the pellet was resuspended in 50 ml extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2% DoDM, and protease inhibitors). After spining at 10 000 *g* for 2 h at 4°C, 1 ml Ni-NTA agarose pre-equilibrated with extraction buffer was added to the supernatant. The mixture was gently agitated at 4°C overnight. After washing three times with 10 ml washing buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM imidazole, and 0.2 mM PMSF), Gβγ proteins were eluted with 10 ml elution buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, and 200 mM imidazole).

### *Direct activation of c-Src by $\beta_2$-AR*

A 125 ng portion of c-Src and 1 μg $\beta_2$-AR with 10 μM isoproterenol or 10 μM alprenolol in 24 μl kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 5 mM $MnCl_2$, and 0.02% DoDM) were incubated on ice for 30 min. ATP (200 μM, final concentration) was added and the mixture was incubated at 30°C for the time period indicated. The reaction was stopped by adding SDS sample loading buffer and incubated at 37°C for 30 min. After 10% SDS–PAGE, anti-phosphotyrosine antibody was used to detect c-Src autophosphorylation.

### *[$^{35}$S]GTPγS loading assay*

The [$^{35}$S]GTPγS loading assay was performed as described previously (Ma *et al*, 2000). $G\alpha_s$ (20 nM), Gβγ (1 μM), and $\beta_2$-AR (30 nM) together with 10 μM alprenolol or isoproterenol in 200 μl loading buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.02% DoDM, and 5 μM GDP) were incubated on ice for 10 min. After incubation at 30°C for 5 min, 100 nM [$^{35}$S]GTPγS was added. At various time points, 40 μl aliquots were moved into 1 ml ice-cold termination buffer (20 mM Tris, pH 8.0, 100 mM NaCl, and 25 mM $MgCl_2$) and loaded onto nitrocellulose membrane (Schleicher&Schuell BioScience). After three washes with 1 ml termination buffer, 4 ml scintillation solution was added to the membrane and $^{35}$S was counted to measure GTPγS loading. GDP (5 mM) was used to determine the nonspecific binding.

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